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Relaxin-Expressing, Fiber Chimeric Oncolytic Adenovirus Prolongs Survival of Tumor-Bearing Mice

Shanthi Ganesh,¹ Melissa Gonzalez Edick,¹ Neeraja Idamakanti,¹ Marina Abramova,¹ Melinda VanRoey,¹ Michael Robinson,¹ Chae-Ok Yun,² and Karin Jooss¹

¹Cell Genesys, Inc., South San Francisco, California and ²Yonsei Cancer Center, Yonsei University College of Medicine, Seoul, Korea

Abstract

Selective replication of oncolytic viruses in tumor cells provides a promising approach for the treatment of human cancers. One of the limitations observed with oncolytic viruses currently used in the treatment of solid tumors is the inefficient spread of virus throughout the tumor mass following intratumoral injection. Data are presented showing that oncolytic adenoviruses expressing the *relaxin* gene and containing an Ad5/Ad35 chimeric fiber showed significantly enhanced transduction and increased virus spread throughout the tumor when compared with non-relaxin-expressing, Ad5-based viruses. The increased spread of such viruses throughout tumors correlated well with improved antitumor efficacy and overall survival in two highly metastatic tumor models. Furthermore, nonreplicating viruses expressing relaxin did not increase metastases, suggesting that high level expression of relaxin will not enhance metastatic spread of tumors. In summary, the data show that relaxin may play a role in rearranging matrix components within tumors, which helps recombinant oncolytic adenoviruses to spread effectively throughout the tumor mass and thereby increase the extent of viral replication within the tumor. Expressing relaxin from Ad5/Ad35 fiber chimeric adenoviruses may prove a potent and novel approach to treating patients with cancer. [Cancer Res 2007;67(9):4399–407]

Introduction

Oncolytic adenovirus therapies use a unique method of tumor destruction by selective viral replication and lysis of tumor cells and therefore represent a novel approach for treating solid tumors (1). The antitumor efficacy of these oncolytic adenoviruses is, however, frequently limited due to variable expression levels of the coxsackievirus-adenovirus receptor (CAR) on tumor cells (2–5), a cell surface receptor used by Ad5-based viruses to bind and enter cells (6). By switching the Ad5 fiber knob with the Ad35 knob that recognizes the highly abundant CD46 receptor on tumor cells (7), viral entry was significantly improved and correlated well with enhanced antitumor activity of Ad5/35 chimeric viruses when compared with Ad5-based viruses (6–8). Although the fiber chimeric viruses significantly increase virus entry into tumor cells, the inability of the virus to efficiently spread throughout a big tumor mass still remains a major obstacle to the development of

a successful adenovirus-based cancer gene therapy for treating solid tumors (9). Recent evidence has shown that connective tissue and extracellular matrix (ECM) components may play a prominent role in inhibiting viral spread following administration of adenovirus (1, 9, 10), and data suggest that, when ECM is degraded by proteolytic enzymes, such as trypsin or collagenase, an enhancement of viral distribution within the tumor can be observed (11, 12).

Relaxin, a peptide hormone, is involved in softening the uterine cervix, vagina, and interpubic ligaments by remodeling or degrading collagen and altering the connective tissue matrix composition in preparation for mammalian parturition (13–16). In addition, relaxin is also known to up-regulate matrix metalloproteinases (17–20). Based on the important role of relaxin as a matrix degradative protein, it is hypothesized that relaxin might facilitate spread of adenovirus within tumors due to the removal of the ECM that may act as a naturally occurring structural barrier to virus spread within tumors.

With the aim of improving cell transduction and viral spread of oncolytic adenoviral vectors within tumors, two relaxin-expressing, replication-competent vectors were generated that contained either the wild-type Ad5 fiber or the Ad5/35 chimeric fiber. These viruses were evaluated for their antitumor efficacy and their effect on metastatic spread of primary tumors and compared with the non-relaxin-expressing vectors (6).

The studies show that relaxin expression enhanced the spread of virus in tumors, in which virus transduction of the parental, non-relaxin-expressing virus was low overall. In addition, relaxin expression also controlled metastatic spread of tumor cells and significantly improved overall survival in tumor-bearing animals. The relaxin-dependent increase in spread of virus was due to the degradation and/or modification of the ECM within the tumor environment.

In summary, Ad5/Ad35 fiber chimeric oncolytic viruses expressing relaxin prolong the survival of tumor-bearing animals by efficiently entering tumor cells and effectively spreading throughout tumors when compared with Ad5-based oncolytic vectors not expressing relaxin and may therefore offer a novel and improved oncolytic therapy for patients with cancer.

Materials and Methods

Construction of relaxin-expressing oncolytic viruses. To generate relaxin-expressing oncolytic viruses, a shuttle plasmid, CP1730, was constructed as described below. The *relaxin-1* gene was amplified by PCR using two primers (5'-AACAGGAGGAGACGACTGACCATGGAGAGCCG-CAGTCAGATC-3' and 5'-GCTTTATTATTTTTTTTATCAGTCTGAGT-CAGGCCCTTCTG-3') and a plasmid (pDNR-LIB-RLX; ref. 21). pDr21F, a plasmid containing the right end of the Ad5 genome, was digested with *Xho*I and *Sph*I, and the 11-kb fragment was gel purified and ligated with the PCR product to generate CP1730. To insert the *granulocyte macrophage colony-stimulating factor* (*GM-CSF*) gene, CP1730 was digested with *Xho*I

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Requests for reprints: Shanthi Ganesh, Cell Genesys, Inc., 500 Forbes Boulevard, South San Francisco, CA 94080. Phone: 650-266-2925; E-mail: shanthi.ganesh@cellgenesys.com.

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and *SpeI*, and the purified 9.9-kb fragment was ligated with the 2,667-bp fragment of CG0070 viral DNA (22) that had been digested with the same enzymes to generate CP1734. This plasmid was digested with *StuI*, *AatII*, and *PacI*, and the purified 6,557-bp fragment was combined with Srf-1-linearized pFLAr20hGM (23) to generate the full-length plasmid CP1740. The same fragment was also combined with Srf-1-linearized pFLAr20hGM-5T35H (6) to generate the fiber chimeric full-length plasmid CP1741. Full-length plasmids were digested with *I-SceI* and transfected into AE1-2a cells (24) to generate the OV-RLX-5 and OV-RLX-5T35H viruses. High-titer virus stocks of relaxin and non-relaxin-expressing oncolytic viruses were generated [7×10^{12} to 8×10^{12} viral particles (vp)/mL] in AE1-2a cells with a particle to plaque-forming unit (pfu) ratio in the range of 15 to 20 for all viruses (25).

Construction of E1-deficient, relaxin-expressing vectors. To generate these viruses, a shuttle plasmid, pAd5L&RSma1-CMV-RLX, was constructed as described below. The *relaxin-1* gene was amplified by PCR using two primers (5'-GTTTAAACATGCCTCGCTG-3' and 5'-GGATCCT-CAGCAATATTAG-3'). The plasmid, pAd5L&RSma1-CMV-GFP (6), was digested with *PmeI* and *BamHI*, and the purified fragment was ligated with the PCR product to generate pAd5L&RSma1-CMV-RLX. Combining Sma1-linearized pAd5L&RSma1-CMV-RLX and the viral DNA of Ad5GFP and Ad5GFP-5T35H (6) generated the full-length plasmids pFLAd5CMV-RLX and pFLAd5/35CMV-RLX. Ad5RLX and Ad5/35RLX viruses were generated by digesting the full-length plasmids with *I-SceI* and transfecting into AE1-2a cells (23). Nonreplicating viruses used in this study were produced (3×10^{11} to 5×10^{11} vp/mL) with the vp to pfu ratio in the range of 15 to 20.

Tumor cell lines. The tumor cell lines used in the study were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Human prostate carcinoma PC-3 cells were purchased from the American Type Culture Collection, and these cell lines were modified to express luciferase to generate PC-3.luc (26). A375-mln1.luc is a cell line established by *in vivo* selection of lymph node metastases from A375 s.c tumor-bearing mice (27).

Western blot analysis of relaxin protein expression. To determine whether the relaxin-expressing replicating and nonreplicating viruses express the *relaxin* gene following gene transfer into PC-3.luc and A375-mln1.luc cells, Western blot analyses were done. Cells were infected with OV-5, OV-RLX-5, OV-5T35H, OV-RLX-5T35H, Ad5RLX, and Ad5/35RLX viruses at 200 vp/cell for 36 h. Out of 1 mL supernatant collected, 8 μ L from Ad5RLX- and Ad5/35RLX-infected cells and 25 μ L from OV-infected cells were loaded and run on a 4% to 12% Bis-Tris gel under reducing conditions and transferred to Invitrolon polyvinylidene difluoride membranes (Invitrogen). Colorimetric detection with rabbit polyclonal antibody raised

against full-length human relaxin (1:500; Santa Cruz Biotechnology, Inc.) and horseradish peroxidase anti-rabbit IgG conjugate antibody (1:5,000; Santa Cruz Biotechnology) identified the 18-kDa, relaxin-specific band.

Invasion assays. Invasion was measured by assessment of the PC-3 cell migration rate through an artificial basement membrane in an invasion chamber (Chemicon International) as described previously (28–30). PC-3 cells were seeded in six-well tissue culture plates at 5×10^5 cells per well. The next day, cells were infected with oncolytic viruses at a given dose. After 3 h of incubation, infection medium was removed and replaced with 3 mL RPMI 1640 containing 10% FBS growth medium, and cultures were incubated at 37°C for 24 h. After the infection, cells were harvested and cell suspensions were prepared (1×10^6 cells/mL) in serum-free medium. After rehydrating the interior of the inserts with serum-free medium for 2 h, it was carefully removed and 500 μ L of medium containing 10% FBS were added to the lower chamber. Cell suspension (300 μ L) from the infections was then added to the interior of the inserts. The inserts were incubated for various durations, and the noninvaded cells and ECM were removed with a cotton swab. The level of cell invasion was quantified by staining the cells on the underlying surface of the invasion membrane with crystal violet and counting them.

In vivo efficacy studies. Female NCR (*nu/nu*) mice (4–6 weeks of age; body weight of 18–20 g) were purchased from Simonsen Laboratories. Mice were injected s.c. in the right flank with either 5×10^6 PC-3.luc cells (26) or 2×10^6 A375-mln1.luc cells (injection volume of 100 μ L; ref. 27). When tumors reached the required mean tumor volume ($100\text{--}125\text{ mm}^3$) as determined by the formula volume = $W \times (L)^2 / 2$ (W is the width and L is the length, in cubic millimeters), animals were randomly distributed into treatment groups and received a total of four intratumoral injections, given once every other day, of 1×10^{10} vps or PBS in a 50 μ L dose volume. Animals from each group were selected at various time points following the initial treatment, bled, and euthanized, and tumors were collected. Tumors were cut into halves, with one half used to detect virus replication and the other half used to determine viral spread within xenografts by immunohistochemistry (6) and to measure the collagen content by trichrome blue staining (21, 31).

For histologic examination, 5- μ m paraffin sections of the tumor tissue were stained for hexon protein using the Zymed's Histomouse kit following the manufacturer's instructions. The remaining half of the tumor was used to isolate virus, and the titer was determined by infecting AE1-2a cells with the extracted virus. Six lymph nodes, including the axillary and inguinal nodes and the lungs, were collected at the selected time points, microinjected with 15 mg/mL luciferin substrate, and imaged after 2 min using the Xenogen imaging system (Xenogen Corp.) to detect luciferase-positive metastases (27).

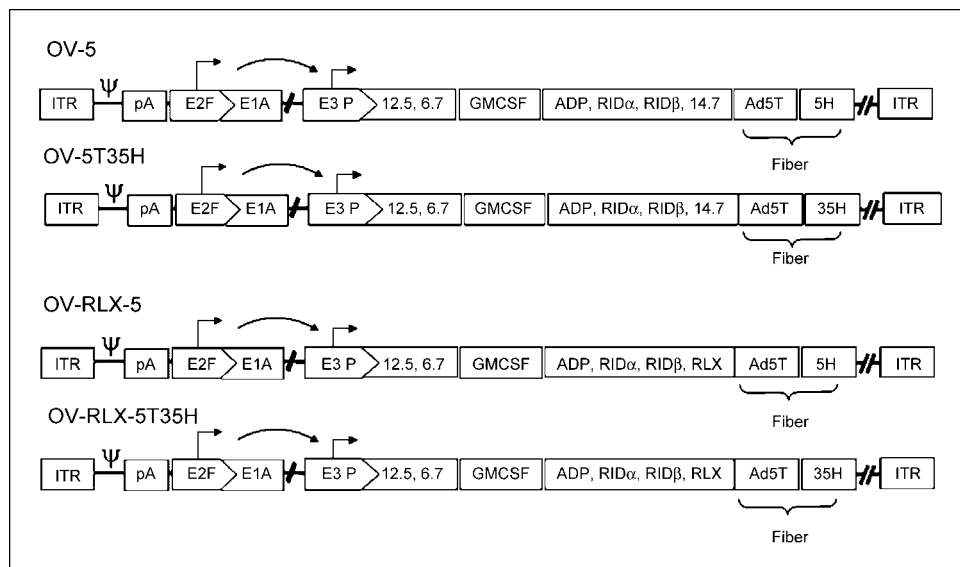


Figure 1. Schematic representation of non-relaxin-expressing (OV-5 and OV-5T35H) and relaxin-expressing oncolytic adenoviruses (OV-RLX-5 and OV-RLX-5T35H). The relaxin-expressing viruses have a gene encoding human relaxin-1 replacing gp 14.7 kDa of E3 region. All four viruses have the gene encoding human GM-CSF replacing gp 19 kDa of the E3 region and the SV40 poly(pA) signal upstream of the tumor-specific promoter driving E1a expression. The expression of E1a in all of these viruses is placed under the control of the E2F-1 promoter, and OV-5 and OV-RLX-5 contain the wild-type Ad5 fiber, whereas OV-5T35H and OV-RLX-5T35H contain the Ad5 shaft and Ad35 knob.

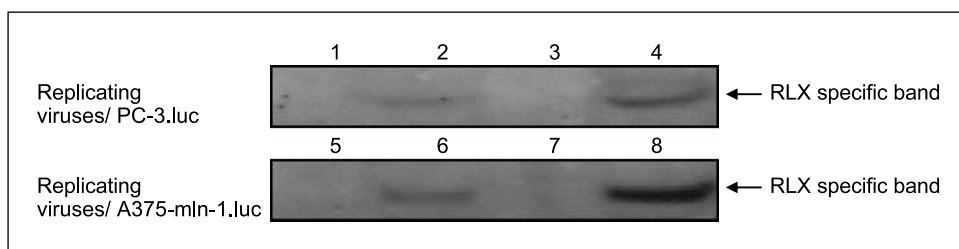


Figure 2. Relaxin (RLX) expression from oncolytic adenoviral vectors. PC-3.luc cells (lanes 1–4) or A375-mln1.luc cells (lanes 5–8) were infected with the relaxin-expressing and non-relaxin-expressing oncolytic vectors at a MOI of 200 vp/cell. At 36 h after infection, supernatants were harvested and 25 μ L were loaded onto an 8% to 12% Bis-Tris polyacrylamide gel and transferred onto a nitrocellulose membrane. Relaxin expression was determined by probing the membrane with a polyclonal, relaxin-specific antibody. An 18-kDa, relaxin-specific band was found for OV-RLX-5 in PC-3.luc (lane 2) and A375-mln1.luc (lane 6) cells and for OV5-RLX-5T35H in PC-3.luc and A375-mln1.luc cells (lanes 4 and 8, respectively). No expression was observed from the non-relaxin-expressing vectors OV-5 or OV-5T35H in PC-3.luc cells (lanes 1 and 3, respectively) or A375-mln1.luc cells (lanes 5 and 7, respectively).

Results

Construction of relaxin-expressing oncolytic adenoviruses.

The parental Ad5 and fiber chimeric Ad5/35 oncolytic adenoviruses carrying relaxin were constructed to study the effect of relaxin expression on efficiency of virus transduction as well as spread and overall virus potency in tumor models. The non-relaxin-expressing parental oncolytic vectors OV-5 and OV-5T35H (6) were engineered to express relaxin by insertion of the human relaxin-1 cDNA in place of the 14.7-kDa gene in the E3 region, generating OV-RLX-5 and OV-RLX-5T35H, respectively (Fig. 1). In all the viruses, the tumor-specific E2F-1 promoter replaced the native E1a promoter to restrict viral replication to cells that have defects in the pRb pathway (32–34). In addition, the cDNA encoding human GM-CSF (hGM-CSF) replaced the open reading frame encoding the 19-kDa glycoprotein of the E3 region (6). The presence of the hGM-CSF gene in these viruses does not alter the relative potency of these vectors in the studies reported here because human GM-CSF is not active in mice and, in addition, all of the studies are done in immunodeficient mice.

Expression of relaxin from OV-RLX-5 and OV-RLX-5T35H virus-infected A375-mln1.luc and PC-3.luc cells was confirmed by an 18-kDa, relaxin-specific band in the Western blots (Fig. 2). Higher intensity bands with the relaxin-expressing chimeric virus OV-RLX-5T35H, compared with the relaxin-expressing Ad5-based virus OV-RLX-5, in both cell lines confirm the higher transduction efficiency of tumor cell lines by the fiber-modified viruses.

Relaxin-expressing oncolytic adenoviruses enhance the *in vitro* invasiveness of PC-3 cells. To test the potential for increasing metastatic events by the expression of relaxin in cells, an *in vitro* cell invasion model system was used (18, 35, 36).

In this model, cell invasion is evaluated by determining the number of cells that migrate from one chamber to another through an ECM-based membrane. The effect of relaxin expression on the level of cell invasion was determined using PC-3 cells infected with OV-5 or OV-RLX-5 at 50 vp/cell for 24 h and loaded into the chamber above the ECM membrane. After 16 h, the number of cells that had migrated through the membrane was determined. Although the frequency of cell invasion in PC-3 cells infected with OV-5 was identical to uninfected PC-3 cells, at least twice as many cells infected with OV-RLX-5 migrated to the lower chamber (Fig. 3A), suggesting that the expression of relaxin can increase the invasiveness of these tumor cells in this *in vitro* model system. Due to the high infectivity of the chimeric viruses, PC-3 cells were infected with these viruses with a multiplicity of infection (MOI) of 1. Under these conditions, the level of invasion of OV-RLX-5T35H–

infected cells was again twice that of the OV-5T35H–infected cells (Fig. 3B), confirming that relaxin expression in PC-3 cells can increase the invasiveness of these cells *in vitro*. In summary, the increased invasiveness of relaxin-expressing PC-3 cells *in vitro* supports the hypothesis that relaxin may degrade ECM components, which may lead to a potential increase in metastatic events *in vivo*.

Efficacy of relaxin-expressing viruses in A375-mln1.luc tumor-bearing mice. To determine whether the ability of relaxin to degrade the ECM enhances virus spread and therefore improves antitumor efficacy in tumor-bearing mice, relaxin-expressing and non-relaxin-expressing viruses were tested in two highly metastatic xenograft models. To test the effect of relaxin in the context of Ad5- and Ad5/35-based oncolytic viruses *in vivo*, the non-relaxin-expressing parental and chimeric viruses, and their corresponding relaxin-expressing viruses, were first tested in a s.c. metastatic A375-mln1.luc xenograft model. The different groups were injected either with PBS or 1×10^{10} vps of OV-5, OV-5T35H, OV-RLX-5, or OV-RLX-5T35H at each injection for a total of four injections, and tumor progression was monitored over time. In the PBS and OV-5 groups, the tumor volume increased 5.3- and 2.4-fold, respectively, over a period of 18 days (Fig. 4A), whereas in the OV-RLX-5–treated mice the tumors hardly progressed during that time, suggesting that the Ad5-based oncolytic virus carrying relaxin was more potent than the Ad5-based nonrelaxin virus. In animals treated with either OV-5T35H or OV-RLX-5T35H, there was minimal tumor progression observed in either group, most likely due to the high transduction efficiency and potency of the non-relaxin-expressing chimeric virus in the A375-mln1.luc model (OV-5T35H, OV-RLX-5, or OV-RLX-5T35H versus OV-5; $P < 0.05$). The difference in transduction efficiency observed between the Ad5-based and the chimeric viruses is due to the different expression patterns of their respective receptors, CAR and CD46, in this model. CD46 expression in the A375-mln1.luc cells is ~ 35 -fold higher than CAR (Supplementary Fig. S1A), resulting in an increased level of virus transduction and overall potency (Supplementary Fig. S1C) of the chimeric vectors when compared with Ad5-based viruses. Therefore, in the A375-mln1.luc model, the expression of relaxin from Ad5-based oncolytic vectors improved the efficacy of the vector, whereas the expression of relaxin did not further enhance the potency of the Ad5/Ad35 chimeric virus due to the high transduction efficiency and overall potency observed with the parental chimeric vector. In summary, the expression of relaxin in the context of an Ad5-based oncolytic virus enhances antitumor efficacy in the A375-mln1.luc tumor

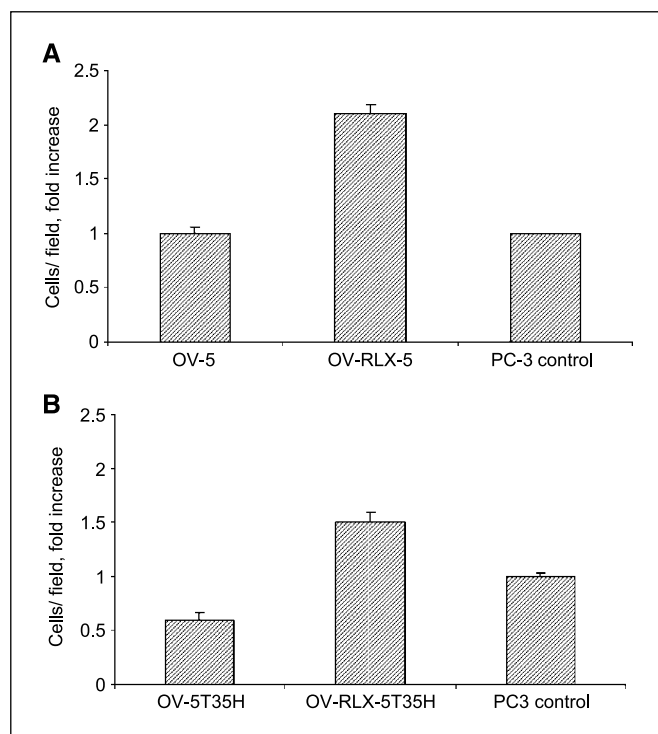


Figure 3. Relaxin expression increases the invasiveness of PC-3 cells in an *in vitro* cell invasion assay. PC-3 cells were infected either with parental (OV-5) or relaxin-expressing Ad5-based oncolytic vectors (OV-RLX-5) at a MOI of 50 vp/cell for 24 h (A) or with parental (OV-5T35H) or relaxin-expressing (OV-RLX-5T35H) chimeric oncolytic vectors at a MOI of 1 vp/cell for 24 h (B). Uninfected PC-3 cells were used as control. Infected or control cells were placed in the upper invasion chamber for 16 h (A) or 8 h (B) for migration through the ECM to occur. Cells that migrated through the ECM were stained with crystal violet, and three high-power fields were counted (40 \times) and plotted as mean cells/field. Columns, mean ($n = 2$); bars, SD.

model, whereas no enhancement of potency was observed in this model when relaxin was expressed from Ad5/Ad35 fiber chimeric, oncolytic viruses.

Enhanced antitumor efficacy of viruses correlates with improved viral spread and replication within tumors. To verify that the increase in therapeutic efficacy with the relaxin-expressing Ad5-based viruses and the chimeric viruses was due to an increase in viral spread and replication, the number of hexon-positive cells and infectious vps within the tumors following virus administration was determined. Tumors were harvested on day 17 following virus injections, and the number of hexon-positive cells was determined by immunohistochemical staining. An increase in hexon-positive cells was found in tumors treated with OV-5T35H and OV-RLX-5T35H (Fig. 4B) compared with tumors treated with OV-RLX-5 and OV-5. The number of hexon-positive cells was slightly lower in the tumors treated with OV-RLX-5 compared with tumors treated with either chimeric vector. There was a further decrease in the number of hexon-positive cells in the tumors treated with OV-5 when compared with the tumors treated with the other OVs. No hexon-positive cells were seen in the control tumors injected with PBS. To further confirm virus replication, a portion of the tumor was used to quantify the infectious vps by titrating tumor homogenates on AE1-2a cells (24). There was no significant difference in the level of infectious virus found in tumors from animals treated with either chimeric virus, and there was a slight decrease (~ 3 - to 5-fold) in the tumors treated with OV-RLX-5 compared with the chimeric

viruses. In contrast, there was a 50-fold drop in the amount of infectious virus in the tumors treated with OV-5 compared with the tumors treated with OV-RLX-5 at day 17 (Fig. 4C). Approximately 2-log more virus was detected in the tumors treated with either chimeric virus when compared with tumors treated with OV-5 (Fig. 4C).

These data suggest that the level of virus spread and infectious particles found in the A375-mln1.luc tumors following treatment with these viruses correlate well with the antitumor efficacy *in vivo* as high levels of virus spread and infectious particles present in tumors result in minimal tumor progression. The data also suggest that, in the context of Ad5-based vectors, the expression of relaxin significantly increases the level of viral spread and overall potency of the vector in this particular model. However, for the chimeric virus platform, the expression of relaxin did not enhance the already potent antitumor activity of the virus due to the high infectivity of the chimeric viruses in the A375-mln1.luc tumor model.

Relaxin expression from adenoviral vectors does not enhance the metastatic events in mice bearing highly metastatic A375-mln1.luc tumors. To evaluate whether the increased invasiveness of relaxin-expressing cells *in vitro* would translate into potentially enhanced metastatic spread of tumors *in vivo*, the formation of metastases in animals treated with the oncolytic viruses in the study described above was monitored. Mice were sacrificed on study day 12, and the presence of tumor cells in lymph nodes and lungs was determined by evaluating the level of luciferase activity in the pooled organs after direct injection with luciferin. The average total photon counts observed in lymph nodes and lungs in the oncolytic virus-treated groups were greatly reduced when compared with organs from PBS-treated animals (Fig. 4D), indicating that the metastatic spread of tumor cells was controlled in virus-treated animals. There was an ~ 3 -fold reduction in the photon counts in the OV-5-treated group compared with the PBS group. In OV-RLX-5-treated and OV-5T35H-treated animals, an ~ 6 -fold and ~ 2 -fold decrease in photon counts was observed compared with either the PBS-treated or OV-5-treated groups, respectively. The OV-RLX-5T35H-treated mice exhibited the lowest photon counts in lymph nodes and lungs at this time point, ~ 2.5 -fold lower than those from OV-RLX-5-treated and OV-5T35H-treated mice. Thus, the relaxin-expressing, vector-treated groups had a reduction in the level of metastasis compared with their parental, non-relaxin-expressing, virus-treated groups. In summary, the *in vitro* data showing an increase in the invasiveness of tumor cells do not correlate with the decrease in the level of metastasis seen *in vivo*.

To further investigate whether high level expression of relaxin within tumors without the oncolytic activity would increase the metastatic events in tumor-bearing mice and therefore pose a potential safety risk in the use of such viruses, parental and relaxin-expressing nonreplicating viruses Ad5GFP, Ad5/35GFP, Ad5RLX, and Ad5/35RLX were tested in the A375-mln1.luc model using the same virus doses and treatment schedules as described above. The Western blot analysis of supernatants from virus-infected cells confirmed the presence of the 18-kDa, relaxin-specific band in both cell lines (Fig. 5B). Higher intensity bands with Ad5/35RLX, compared with the Ad5RLX, in both cell lines again confirmed the higher transduction efficiency of these tumor cell lines by the fiber-modified viruses. Furthermore, higher intensity of relaxin-specific bands was also observed with all of the relaxin-expressing nonreplicating viruses compared with the replicating viruses due to

the use of the strong constitutive cytomegalovirus (CMV) promoter driving relaxin expression in these viruses (Figs. 2 and 5B).

No antitumor efficacy was observed with any of the non-replicating viruses (Fig. 5C) in the A375-mln1.luc model, indicating that the relaxin protein itself has no antitumor effect that translates into a reduced tumor growth rate. Selected mice ($n = 3$) were sacrificed on study day 12 to monitor metastasis in lymph nodes and lungs. All the mice in the groups treated with the non-replicating viruses were positive for luciferase activity, and the average total photon counts of all those groups were similar to the photon counts of PBS-injected control animals (Fig. 5D), suggesting that the high level expression of relaxin intratumorally in the absence of any oncolytic activity does not increase the metastatic potential of the tumor in the highly metastatic A375-mln1.luc model. In summary, although the expression of relaxin increased the level of tumor cell invasion in an *in vitro* assay, relaxin-expressing oncolytic adenoviruses and non-relaxin-expressing, fiber chimeric oncolytic viruses decreased the number of metastases at distant sites in a highly metastatic tumor model *in vivo*, suggesting that the increased potency of these viruses in controlling tumor growth, as observed earlier, probably leads to a reduction in metastasis at distant sites. The results also show that the viruses expressing relaxin without the capability to replicate or kill tumor cells do not increase the metastatic events, although those nonreplicating viruses expressed very high levels of relaxin

in vitro and did not control growth of the primary tumors (Figs. 2 and 5B).

Relaxin expression enhances the antitumor efficacy of fiber chimeric viruses in PC-3.luc tumor-bearing mice. The relaxin-expressing and non-relaxin-expressing chimeric oncolytic viruses were tested further in the highly metastatic PC-3.luc xenograft model. This model was specifically chosen for evaluating the potency of the chimeric oncolytic viruses because of the reduced transduction efficiency of PC-3.luc cells by these vectors compared with A375-mln1.luc cells. The reduced transduction efficiency offered the potential of detecting a benefit by expressing relaxin in the context of chimeric oncolytic viruses as had been observed with the Ad5-based viruses in the A375-mln1.luc model. Although CD46 receptors are highly expressed in both A375-mln1.luc and PC-3.luc cells (Supplementary Fig. S1A and B), the transduction of chimeric virus was only 50% in PC-3.luc cells compared with >90% in A375-mln1.luc cells at a MOI of 50 (Supplementary Fig. S1A and B). This is most likely due to very low level expression of the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ found in PC-3.luc cells, which serve as coreceptors for internalization of virus bound to the cell surface receptor (Supplementary Fig. S1A and B). The high level expression of CARs found in PC-3.luc cells compared with the A375-mln1.luc cells resulted in the Ad5-based vector being almost as potent in this model as the chimeric virus (Supplementary Fig. S1D). Thus, only the chimeric viruses were tested in the PC-3.luc model. Tumors

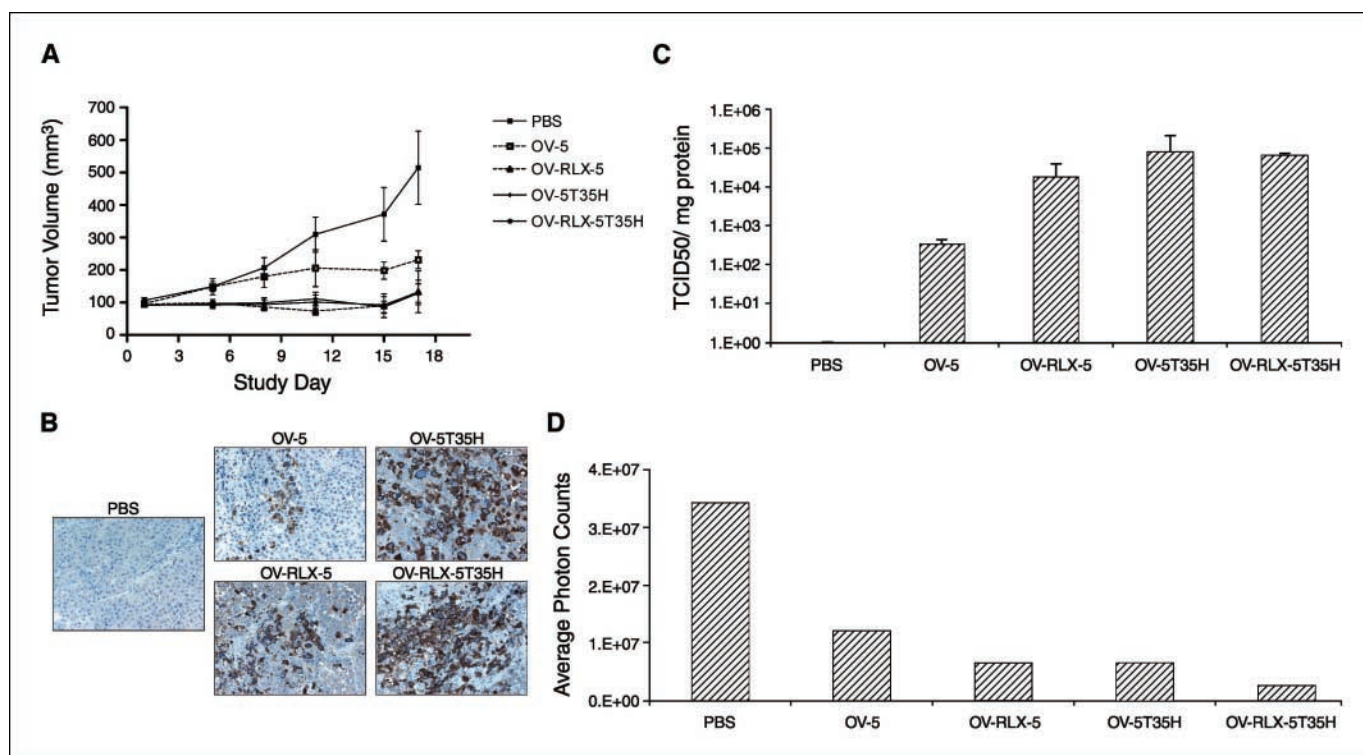


Figure 4. Enhanced antitumor efficacy of relaxin-expressing vectors in an A375 metastatic melanoma model. Nude mice bearing s.c. A375-mln1.luc tumors were injected intratumorally when the average tumor size reached $\sim 100 \text{ mm}^3$ with either PBS, relaxin-expressing (OV-RLX-5 and OV-RLX-5T35H), or parental oncolytic viruses (OV-5 and OV-5T35H) with $1 \times 10^{10} \text{ vp/mouse/injection}$ once every other day for a total of four injections, and the tumor volume (A) was determined by caliper measurement. Points, mean tumor volume (mm^3 ; $n = 10$ per group); bars, SE. A statistically significant delay in tumor progression was observed between all vector-treated groups compared with PBS-injected mice (OV-5, $P < 0.01$; OV-5T35H, OV-5T35H, and OV-RLX-5T35H, $P < 0.0001$) and between relaxin-expressing vectors (OV-5-RLX and OV-RLX-5T35H) compared with the Ad5 parental vector (OV-5, $P < 0.05$). Seventeen days following the initial viral injection, tumors were harvested ($n = 3$) and analyzed for virus spread by determining the number of hexon-positive cells by immunohistochemistry on paraffin-embedded tumor tissue (B) and for the amount of infectious virus by titrating tumor lysates on AE1-2A cells (C). The level of metastasis after virus treatment was evaluated in animals ($n = 3$) on day 12 after virus injections (D) by isolating lungs and selected lymph nodes and injecting them with luciferin to determine photon counts using the Xenogen system. Level of metastasis as indicated by the combined average total photon counts from lungs and lymph nodes.

were injected with PBS, OV-5T35H, or OV-RLX-5T35H with the same doses and treatment schedules as described above, and the growth of the primary tumor as well as the formation of metastases in lymph nodes and lungs were monitored.

Within 26 days after the first viral injections, tumors of PBS-treated animals increased 8-fold in size (Fig. 6A) in contrast to tumors from OV-RLX-5T35H-treated animals, which had only increased 1.6-fold. A slight but significant ($P < 0.0001$) increase in tumor burden (4.2-fold) was noted in mice treated with OV-5T35H when compared with the OV-RLX-5T35H-treated group. These data suggest that relaxin expression from fiber chimeric oncolytic viruses can lead to enhanced potency when compared with non-relaxin-expressing, fiber chimeric viruses in models in which the initial transduction of these viruses is limited.

Disruption of the collagen network within tumors enhances virus spread. To evaluate whether the increase in therapeutic efficacy of OV-RLX-5T35H in the PC-3.luc model was due to an increase in virus spread, tumors were harvested on day 28 following initial virus injection, and the number of hexon-positive cells as well as the amount of infectious vps present in the tumor were determined as described above. In the PC-3.luc tumors treated with OV-RLX-5T35H, there was an increase in the number of hexon-positive cells compared with OV-5T35H-injected tumors (Fig. 6B, top). When the tumors were stained with Mason's

trichrome blue, which stains collagen (21, 31, 37), reduced staining was observed in tumors treated with the relaxin-expressing chimeric virus compared with the parental chimeric virus or the PBS-injected tumors (Fig. 6B, bottom), suggesting that there might be a relaxin-mediated alteration in the collagen bundles or a reduction in the total collagen amount. When the tumor homogenates were titrated on AE1-2a cells (24), there was an ~5-fold increase in the level of infectious virus present in PC-3.luc tumors treated with OV-RLX-5T35H compared with tumors from animals treated with OV-5T35H (data not shown). These data suggest that chimeric oncolytic vectors expressing relaxin show better antitumor potency than non-relaxin-expressing chimeric oncolytic vectors by modifying or degrading the tumor matrix and therefore enhancing overall virus spread within tumors.

Relaxin-expressing chimeric oncolytic viruses control metastases in PC-3.luc tumor-bearing mice leading to prolonged overall survival. The performance of these chimeric oncolytic viruses was further evaluated in this model by measuring metastases in lymph nodes and lungs. PC-3.luc tumors were injected with the same virus doses and treatment schedules as described above. Forty-two days after the initial viral injection (study day 42), the percentage of mice with metastases in either lymph nodes or lungs in PBS-treated, OV-5T35H-treated, and

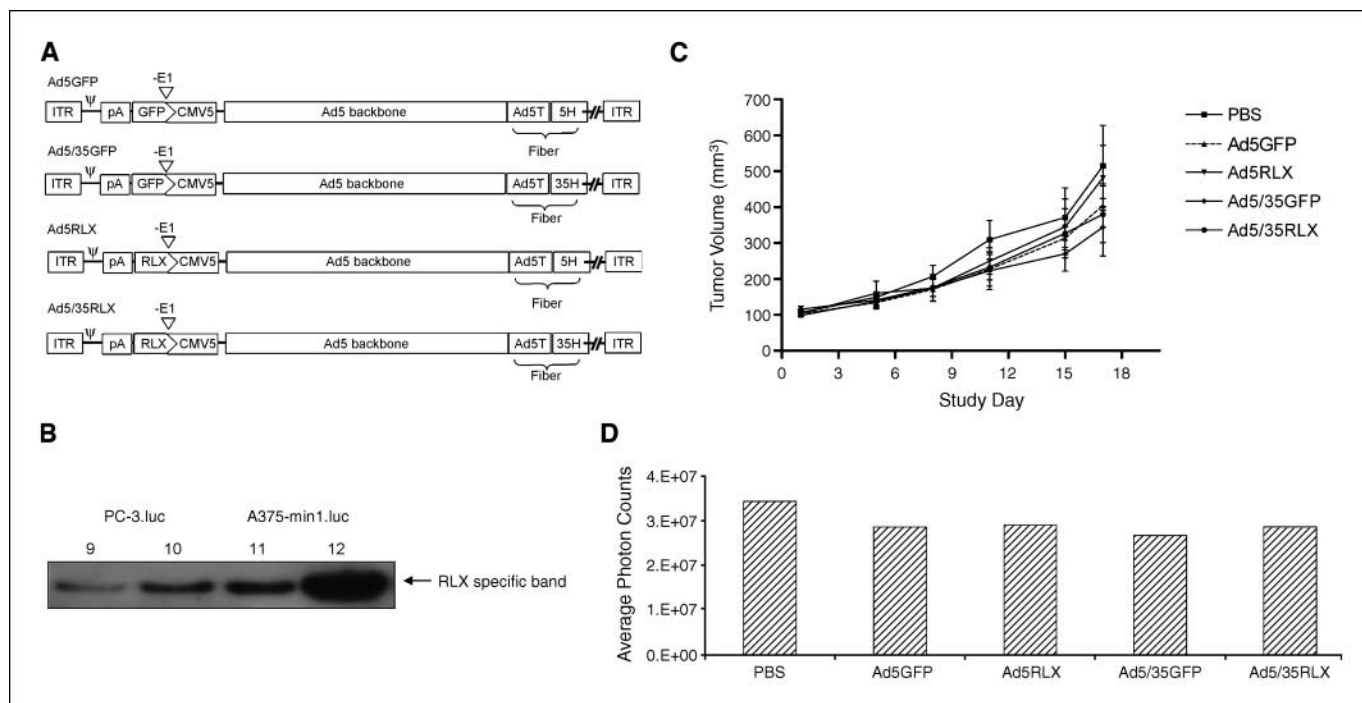


Figure 5. Relaxin-expressing, replication-deficient adenovirus vectors do not enhance metastatic spread of A375-mn1.luc tumors. **A**, a green fluorescent protein (GFP) reporter gene under the control of a strong constitutive CMV promoter was incorporated into the E1 region of adenoviral vectors that contained the fiber knob from either Ad5 (Ad5GFP) or Ad35 (Ad5/35GFP) adenovirus. The GFP reporter gene was replaced with the human *relaxin-1* gene to generate the relaxin-expressing nonreplicating viruses with the Ad5 fiber (Ad5RLX) or the Ad5/35 fiber (Ad5/35RLX). **B**, the level of relaxin expression from these nonreplicating adenoviral vectors was determined by Western blot analysis. PC-3.luc cells or A375-mn1.luc cells were infected with the relaxin-expressing nonreplicating viruses at a MOI of 200 vp/cell for 36 h, and 8 μ L of the supernatants were loaded onto an 8% to 12% Bis-Tris polyacrylamide gel that was transferred to a nitrocellulose membrane. Relaxin protein was detected as described in the legend of Fig. 2. The relaxin-specific, 18-kDa band was observed for Ad5RLX in PC-3.luc (lane 9) and A375-mn1.luc (lane 11) cells and for Ad5/35RLX in PC-3.luc and A375-mn1.luc cells (lanes 10 and 12, respectively). To evaluate the antitumor efficacy of these viruses in the A375-mn1.luc model, nude mice bearing s.c. A375-mn1.luc tumors ($n = 10$) were injected intratumorally with PBS or relaxin or the non-relaxin-expressing nonreplicating viruses (Ad5RLX, Ad5/35RLX, Ad5GFP, and Ad5/35GFP) with 1×10^{10} vp/mouse/injection once every other day for a total of four injections. Tumor volume (**C**) was determined by caliper measurement. Points, mean tumor volume (mm³; $n = 10$ per group); bars, SE. The level of metastasis was evaluated on day 12 after virus injections (**D**) by isolating lungs and selected lymph nodes and injecting them with luciferin to determine photon counts using the Xenogen system. Combined total average photon counts from the lungs and lymph nodes ($n = 3$).

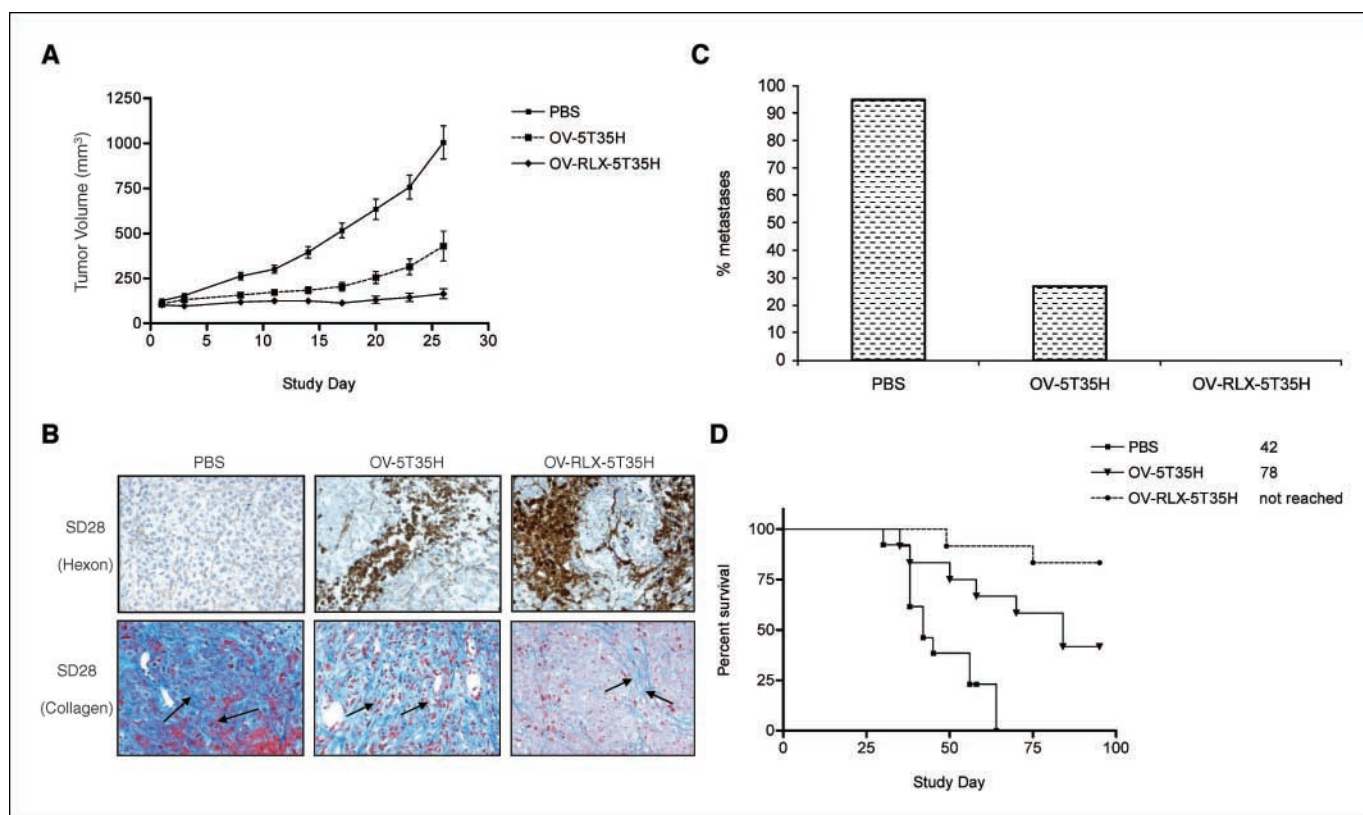


Figure 6. Relaxin expression increases the antitumor efficacy of fiber chimeric viruses in a spontaneous metastatic prostate cancer (PC-3.luc) model. Nude mice bearing s.c. PC-3.luc tumors ($n = 33$) were injected intratumorally with PBS, the parental virus (OV-5T35H), or relaxin-expressing virus (OV-RLX-5T35H) with 1×10^{10} vp/mouse/injection once every other day for a total of four injections. The tumor volume (A) was determined by caliper measurement. Points, mean tumor volume (mm³; $n = 33$ per group); bars, SE. A significant difference in tumor progression was observed between the PBS-treated and vector-treated groups ($P < 0.0001$) and between the parental chimeric (OV-5T35H) and relaxin-expressing chimeric viruses (OV-RLX-5T35H; $P < 0.0001$). B, top, twenty-eight days following the initial viral injections, tumors were harvested and immunohistochemistry was done on tumor sections to determine virus spread by measuring the number of hexon-positive cells; bottom, at the same time point, the level of collagen within the tumor was evaluated by trichrome blue staining. The level of metastasis after virus treatment was evaluated in the animals on day 42 after virus injections (C) as described in the legend of Fig. 5. D, to evaluate the effect of these viruses on overall survival in the PC-3.luc model, animals were treated ($n = 12$) as described above and monitored for survival for up to 95 d.

OV-RLX-5T35H-treated groups was 80, 27, and 0, respectively (Fig. 6C). In summary, relaxin-expressing, chimeric oncolytic adenovirus treatment decreased the number of metastases in lymph nodes and lungs in two highly metastatic tumor models, suggesting that the increased potency of these viruses in controlling growth of the injected primary tumor most likely leads to a reduction in metastasis at distant sites.

To evaluate whether the improved efficacy of these vectors in controlling tumor progression and metastases translates into improved survival, the study was repeated with survival as the end point. PBS-injected animals had a mean survival time (MST) of 42 days with no animals alive by day 62 (Fig. 6D), whereas a significant improvement in survival was observed in the OV-5T35H-treated mice with a MST of 78 days and 42% of animals alive at the end of the study on day 95. A further improvement in survival was observed in the OV-RLX-5T35H-treated group, with the MST not yet reached by day 95 and 83% of the animals alive at that time point. Interestingly, complete tumor regression was noted in 3 of 12 mice treated with either the OV-5T35H or the OV-RLX-5T35H viruses. These survival data confirm that chimeric oncolytic vectors expressing relaxin show greater antitumor efficacy and therefore lead to improved survival of treated animals when compared with non-relaxin-expressing, fiber chimeric oncolytic vectors.

Discussion

The inability of Ad5-based oncolytic viruses to consistently transduce a broad range of tumor cells and efficiently spread throughout solid tumors seems to hamper their effectiveness in clinic (1, 33). A fiber knob replacement strategy, generating viruses with chimeric fibers, has previously been shown to improve the transduction efficiency of such viruses significantly (6). In the study described here, the fiber chimeric viruses were combined with a strategy aiming at increasing spread of viruses within tumors by introducing the gene for a matrix-degrading peptide hormone into these viruses. Because the ECM is known for its role in limiting virus spread throughout tumors (15, 38, 39), the *relaxin* gene was incorporated into the OV backbone to degrade/decrease matrix components following virus infection of tumor cells, thereby potentially improving virus spread throughout the tumor (16, 21). Kim et al.'s recent finding also supports the idea of improving spread with a relaxin-expressing oncolytic virus (21).

Our data suggest that relaxin expression in the context of Ad5-based and fiber chimeric oncolytic viruses enhances the potency of such viruses if it is limited due to poor initial transduction efficiency. Both relaxin-expressing oncolytic viruses and the non-relaxin-expressing, fiber chimeric oncolytic viruses were more efficacious in controlling tumor growth than the non-relaxin-expressing, Ad5-based virus ($P < 0.05$) in A375-mln1.luc model.

Although there was no difference seen in the efficacy between OV-RLX-5T35H and OV-5T35H due to the potent efficacy of the non-relaxin-expressing chimeric vectors in this particular model, the addition of relaxin to the Ad5-based virus resulted in a dramatic increase in potency to a level comparable with the chimeric viruses. This increase in potency was due to an increase in virus spread within tumors as indicated by the increase in hexon-positive cells. The increased virus spread correlated with an increase in the level of infectious virus within the tumor, which in turn led to an improved ability to control tumor growth. In the A375-mln1.luc model, the difference in activity of the fiber chimeric vectors versus Ad5-based viruses is most likely due to the difference in the level of their respective receptors, with the CAR used by Ad5-based viruses low with respect to the CD46 receptor that is used by the Ad5/35 fiber chimeric viruses (7). The difference in the expression of the two receptors correlates with the poor transduction efficiency and overall antitumor potency of Ad5-based viruses and the good transduction efficiency and potency of fiber chimeric viruses in this model.

To determine if the expression of relaxin could increase the potency of fiber chimeric oncolytic vectors in some models, a second metastatic model was tested in which the fiber chimeric, parental vectors showed lower transduction efficiency. In support of the hypothesis described above, the relaxin-expressing, fiber chimeric virus showed significantly better antitumor efficacy in the metastatic PC-3.luc model when compared with the non-relaxin-expressing, fiber chimeric virus ($P < 0.0001$). In this study, metastatic events were greatly reduced with the virus treatment, and unlike in the *in vitro* studies, the metastatic events were further reduced with the relaxin-expressing virus compared with the non-relaxin-expressing, fiber chimeric virus. Interestingly, the increased potency of the relaxin-expressing, fiber chimeric virus in this model correlated with an enhanced degradation and/or structural modification of collagen within the matrix in the relaxin-expressing, virus-treated groups and overall spread of the virus within the tumor mass (40). This modification of the ECM potentially removes some of the structural barriers within the tumor that prevents effective virus spread (16). Kim et al. also showed a reduction in the level of collagen staining within tumors after treatment with relaxin-expressing viruses and proposed that this reaction was due to a degradation of the ECM, which slowed down tumor growth (21).

In the study presented, the increased virus spread, as indicated by the increase in number of hexon-positive cells, allowed a

greater level of virus replication within tumors, which ultimately lead to an increase in antitumor potency. As was observed in the A375-mln1.luc model, the growth rate of the primary tumor and the number of metastases were controlled or reduced in the metastatic PC-3.luc model in the relaxin-expressing, virus-treated animals when compared with animals treated with the non-relaxin-expressing virus, which contradicts with the data we saw in our *in vitro* studies that relaxin expression could increase cell invasion.

This apparent discrepancy could be due to the fact that the *in vitro* assay evaluates only a few steps necessary for metastases to occur (e.g., mobility and invasion of cells), whereas in the *in vivo* models all steps, including dissociation of cells from the primary tumor, enhanced cell motility, and the ability of cells to invade stroma and to grow effectively at distant sites, are required for metastasis to occur (29, 41). If relaxin is able to only affect one or a few of these steps of the complex metastatic process, it might not lead to increased metastatic events in tumor models. Alternatively, due to the enhanced antitumor potency of the relaxin-expressing viruses, these viruses effectively control the growth of the primary tumor and therefore indirectly may control the spread of the tumor cells to distant sites because this process is related to the size of the primary tumor in these models (27). However, the relaxin-expressing, replication-incompetent viruses do not increase the metastasis in a highly metastatic tumor model, suggesting that even strong overexpression of relaxin within growing tumors will not increase their metastatic potential.

In conclusion, the data presented herein suggest that, by replacing the fiber knob of Ad5-based viruses with the Ad35 fiber knob and/or introducing the *relaxin* gene into the oncolytic adenoviral backbone, transduction efficiency of viruses and the spread of the virus within solid tumors can be significantly improved, leading to prolonged survival of tumor-bearing mice. Thus, oncolytic viruses with Ad5/35 fibers, expressing the *relaxin* gene, may offer a novel promising therapy for the treatment of patients with cancer.

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Correction: Adenovirus Prolongs Survival of Tumor-Bearing Mice

In the article on how adenovirus prolongs survival of tumor-bearing mice in the May 1, 2007 issue of *Cancer Research* (1), the Acknowledgments should have read as follows:

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1. Ganesh S, Gonzalez Edick M, Idamakanti N, Abramova M, VanRoey M, Robinson M, Yun C-O, Jooss K. Relaxin-expressing, fiber chimeric oncolytic adenovirus prolongs survival of tumor-bearing mice. *Cancer Res* 2007;67:4399-407.